Genetic transformation of *Pseudomonas oleovorans* by electroporation

Introduction

Pseudomonas oleovorans carries out a wide variety of interesting biological activities, such as the synthesis of a class of biodegradable polymers, the medium-chain-length poly(β-hydroxyalkanoates) (de Smet *et al.*, 1983; Gross *et al.*, 1989; Cromwick *et al.*, 1996); the dissimilation of alkane compounds (Chakrabarty *et al.*, 1973); and the ω-oxidation, hydroxylation and epoxidation of various hydrocarbons, alkenes and fatty acids (Peterson *et al.*, 1966; Schwartz, 1973; Abbott and Hou, 1973; Besse and Veschambre, 1994; Archelas and Furstoss, 1997).

Genetic modification could expand the usefulness of *Ps. oleovorans* in various industrial applications. Previous efforts in the genetic engineering of this organism have used the efficient but complicated conjugational transfer procedures that are often species-specific (Chakrabarty *et al.*, 1973; Steinbüchel and Schubert, 1989). Jahng and Wood (1994) reported an unsuccessful electroporation attempt to transform a *Ps. oleovorans* strain. In this communication, the development of an electrotransformation procedure for this pseudomonad species is described. A 5.9-kb shuttle plasmid (pCN51) that contains pseudomonad pPS10 and *E. coli* pMB9 replicons, and a kanamycin-resistance determinant, was used in this study (Nieto *et al.*, 1990). The electroporation procedure presented in this paper provides for a simple means of genetically modifying *Ps. oleovorans*.

Materials and methods

Bacterial strains, plasmids, and growth conditions

Ps. oleovorans NRRL B-14683 and *Escherichia coli* DH5α were obtained from the NCAUR/ARS/USDA (Peoria, IL) and Life Technologies (Gaithersburg, MD), respectively. Bacteria containing the plasmids pCN51 (Nieto *et al.*, 1990) and pSG312 were purchased from ATCC (Manassas, VA). All microorganisms were grown in Luria medium (1% w/v tryptone; 0.5% w/v yeast extract; 0.5% NaCl). Solid medium was prepared in agar (1.0–1.2%). When needed, ampicillin (100 μg/ml) or kanamycin (30 μg/ml) was included in the culture medium. The pseudomonads and *E. coli* were grown at 30°C and 37°C, respectively.

Molecular biology procedures

Mini-plasmid screening was performed by an alkaline lysis procedure (Sambrook *et al.*, 1989). Large-scale plasmid isolation was carried out by using a Plasmid Midi Kit (Qiagen Inc., Valencia, CA). Restriction enzymes were purchased from Life Technologies and New England Biolabs (Beverly, MA). Agarose gel electrophoresis of DNA was performed in the TBE buffer system (0.089 M Tris base, 0.089 M boric acid, 0.002 M sodium EDTA).

Electroporation

Cells from an overnight culture (50 ml) were harvested by centrifugation (6,500 × g; 15 min; 4°C), washed once in a cold 0.3M sucrose solution (10 ml), and resuspended to a selected cell density in the same solution. The cell suspension (100 μl) was transferred to an electroporation cuvette (Bio-Rad Laboratories, Hercules, CA). A desired amount

of plasmid was added to the cells. Following a 5 min incubation on ice, the mixture was electroporated in a Bio-Rad Gene Pulser II electroporator equipped with a Pulse Controller Plus module. Unless specified otherwise, the variables of the electroporation system were set at 200 ohms, 25 μF and 2.5 kV. The electroporated cells were added to 9× volume of a SOC medium (Hanahan, 1983) and incubated at 30°C for 1–2 hr with 250 rpm rotary shaking. Aliquots of the cell suspension were spread on solid LB medium containing an appropriate antibiotic to select for transformants.

Results and discussion

The effect of cell-growth stage on the efficiency of *Ps. oleovorans* electrotransformation was examined to determine the optimal time to harvest the cells for electroporation. An overnight culture was used to inoculate 50 ml of a fresh LB medium in a 125-ml Erlenmeyer flask at 1/25× dilution. The culture was grown at 30°C with 250 rpm shaking, and sample aliquots were removed at various time points and processed for electroporation as described in **Materials and methods**. The results (Fig. 1) showed that *Ps. oleovorans* was most responsive to electrotransformation within the first hour of culture transfer into a fresh growth medium. The efficiency of transformation rapidly decreased if the culture was allowed to grow for 1.5–2.5 hrs. The overnight culture was completely recalcitrant to transformation under the electroporation conditions. The cell

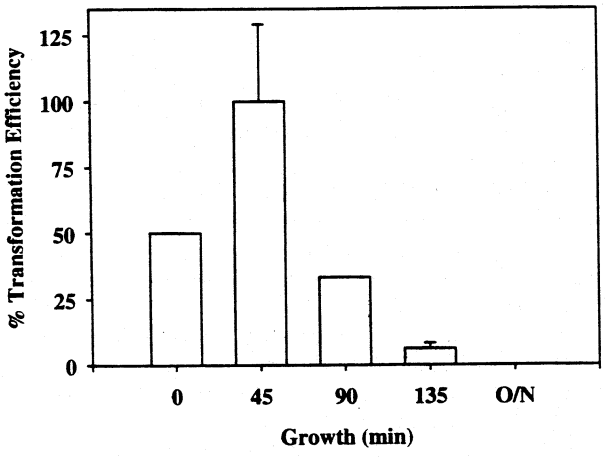

Figure 1 Relative electrotransformation efficiency of *Ps. oleovorans* at various growth stages. Plasmid pCN51 (10μg) was added to a cell suspension (OD_{600nm} = 1.0) in a 0.2-cm gap electroporation cuvette. Electroporation was effected at 200 ohms, 25 μF and 2.5 kV. The measured time-constants were 4.94 to 5 msec. The total number of transformants obtained with the 45-min culture was defined as 100% relative electrotransformation efficiency. O/N = overnight culture.

Table 1 The effect of plasmid DNA concentration on *Ps. oleovorans* electrotransformation. Cell suspension (OD_{600nm} = 10) was placed in a 0.2-cm gap-width cuvette. Electroporation conditions were: 2.5 kV, 25 μF, and 200 Ω. The observed time-constants of electroporation ranged from 4.50 to 5.04 msec. Km^r = kanamycin resistant.

DNA added (μg)	*Total number of Km^r transformants*
1.19	200 (n = 2)
5.95	300–2,000 (n = 5)
11.90	0–400 (n = 4)

morphology may have caused the decrease of electrotransformantion efficiency of the culture grown for 1.5–2.5 hrs, since it was observed on close examination the apparent formation of small spherical structures indicative of cell aggregation. Based on this result, subsequent electroporation experiments were performed with cells harvested within the first hour of a culture inoculation.

The effect of plasmid concentration on the outcome of the electrotransformation was next studied. Table 1 shows that the addition of about 6 μg plasmid DNA yielded the highest number of transformants. A lower plasmid concentration expectedly yielded fewer number of transformants as the amount of DNA was not enough to transform all the available competent cells. Interestingly, increasing the amount of the added DNA to 12 μg not only failed to increase but actually caused a decrease in electrotransformation efficiency. The cause for this apparent decrease of transformation efficiency was not understood. The results nevertheless showed that an overloading of plasmid DNA in an electroporation procedure might indeed be deleterious to the transformation efficiency.

The density of the cell suspension could influence the total number of transformants recoverable from the electroporation protocol. Figure 2 shows that the relative transformation frequency is the highest when the electroporation was performed with a cell suspension having an OD_{600nm} of 5. At a lower cell density, the transformation frequency of *Ps. oleovorans* decreased, possibly due to a higher number of transformants killed by the applied voltage. The lower transformation frequency observed at the highest cell-density used in this experiment, on the other hand, could not be attributed to the insufficient amounts of DNA, since earlier study (Table 1) had shown that a saturation level of the plasmid existed in the current experiment conditions. A possible explanation is that an increased contact or aggregation of cells in the high-density suspen-

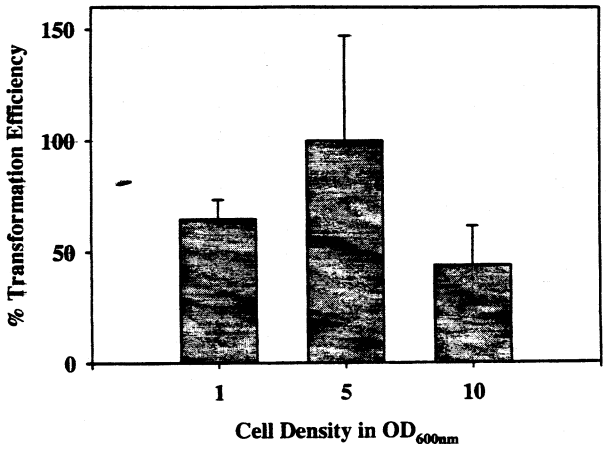

Figure 2 Relative electrotransformation efficiency of *Ps. oleovorans* as a function of cell density. Six micrograms of pCN51 plasmid was added to cell suspensionS of various OD_{600nm} in 0.2-cm gap electroporation cuvettes. Electroporation was effected at 200 ohms, 25 μF and 2.5 kV. The measured time-constants were 4.88–5.02 msec. The total number of transformants obtained with the OD_{600nm} = 5 sample was defined as 100% relative electrotransformation efficiency.

sion impaired the uptake of the transforming plasmid through an unknown mechanism.

The field strength of electroporation influences the survival rate of the cells and the extent of plasmid uptake by the organisms. The gap width of the electroporation cuvette dictates the effective voltage experienced by the cells. As expected, data in Table 2 show that cells exposed to the lower field strength (6.25 and 12.5 kV/cm) exhibited at least a two-fold higher survival rate than those exposed to the higher effective voltage of 25 kV/cm . The highest total number of transformants, however, was obtained with cells electroporated in the 0.1-cm gap-width cuvettes. These data underscore the importance of choosing an electric field that would yield the highest number of the desired transformants without compromising the cell survival rate.

The transformation efficiencies reported for the other species of pseudomonad ranged from 19 transformants/μg pSMMO20 (14.7 kb) plasmid for *Ps. cepacia* G4 strain, to 1.1×10^5 transformants/μg pSUP104 (9.5 kb) or pSR134 (18.6 kb) plasmid in *Ps. putida* PpY101 (Iwasaki *et al.*, 1994; Jahng and Wood, 1994). The highest electroporation efficiency of $(6.9 \pm 2.3) \times 10^2$ transformants/μg pCN51 (5.9 kb) was achieved in this study with the *Ps. oleovorans* cells electroporated in a 0.1-cm gap-width cuvette. This level of efficiency is more than sufficient for an effective genetic manipulation of this important pseudomonad. Iwasaki *et al.* (1994) had shown that the plasmid size did not significantly affect the transformation efficiency. Based on their observation, it is anticipated that pCN51 carrying an exogenous gene can still effectively electrotransform *Ps. oleovorans* using the procedure developed in this study. The electroporation protocol described in this paper thus provides a simple and powerful means for the genetic modification of industrially useful *Ps. oleovorans*.

Table 2 *Ps. oleovorans* survival rate and transformation frequency at various effective voltages of electroporation. A 1-hr culture was harvested and resuspended in 0.3M sucrose solution to an OD_{600nm} of 5. Plasmid pCN51 at 4.1 μg was used in each transformation sample. Electroporation conditions were: 2.50 kV, 25 μF, and 200 Ω. The observed time-constants were 4.78 to 5.12 msec. Cells were recovered in SOC medium at 30°C for 2 hrs with 250 rpm agitation. Serial-dilution plating on LB and direct plating on LB+Kanamycin solid medium were performed to enumerate viable-cell concentrations and total transformants, respectively. Km^r = kanamycin resistant.

Cuvette gap-width (cm)	*Effective voltage (kV/cm)*	*Survival cell density ($\times 10^8$ cfu/ml)*	*Total number of Km^r transformants ($\times 10^3$)*
0.1	25	1.0 ± 0.5 (n = 2)	2.8 ± 0.9 (n = 2)
0.2	12.5	2.3 ± 0.7 (n = 2)	1.7 ± 0.7 (n = 2)
0.4	6.25	2.2 ± 0.2 (n = 2)	0.1 ± 0.1 (n = 2)